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Isodesacetyluvaricin, an Annonaceous Acetogenin, Specifically Inhibits Gene Expression of Cyclooxygenase-2

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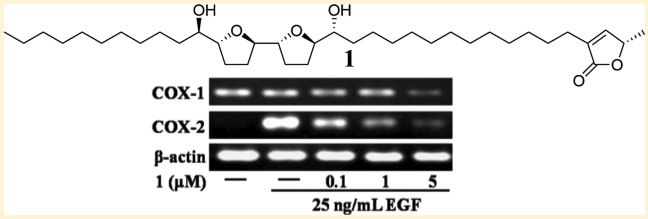
Supporting Information

ABSTRACT: Cyclooxygenase-2 (COX-2) is an inducible isoform of the enzyme responsible for the synthesis of several inflammatory mediators. In a search for phytochemicals with anti-inflammatory activity, the COX-2 inhibitory activity of 15 typical Annonaceous acetogenins was examined. Isodesacetyluvaricin (**1**), from the Formosan tropical fruit tree *Annona glabra*, exhibited the most potent activity. Reverse transcription PCR was used to test the effect of **1** on epidermal growth factor-stimulated expression of COX-2 in cultures of A431 human epidermoid carcinoma cells. Three hours after exposure to **1** (5 μM), A431 cells had barely detectable levels of COX-2 mRNA. A corresponding but smaller decline in the COX-2 protein appeared on using Western blots. Lipopolysaccharide-stimulated expression of COX-2 in Raw 264.7 mouse leukemic monocyte−macrophages showed a similar decrease. Luciferase assays revealed that cells exposed to **1** had reduced activities of two COX-2 promoter-transcription factors: cAMP response element-binding factor and nuclear factor of activated T-cells. Compound **1** did not affect cell proliferation, as measured by a colorimetric assay, or intracellular store-operated calcium influx, as determined by fluorescence imaging. Thus, **1** may serve as a lead compound for targeting inflammatory diseases as well as angiogenesis and cancer metastasis.



In most mammalian cells, cyclooxygenase (COX) is a rate-limiting isoenzyme that catalyzes the synthesis of prostanoids that mediate inflammation. Prostanoids include prostaglandins, thromboxanes, and prostacyclins, which are metabolites of arachidonic acid that cause vasodilation, edema, and pain associated with tissue injury.[1,2] Different types of tissues express two COX protein isoforms in varying amounts. COX-1 is a constitutive enzyme, while COX-2 is inducible. Induction of COX-2 gene expression occurs in response to growth factors, mitogens, and inflammatory cytokines. Overexpression of COX-2 correlates with severe inflammation, angiogenesis, and cancer metastasis.[3,4] Thus, reducing the expression of COX-2 is a major goal of pharmacological treatment strategies.[5,6]

Annonaceous acetogenins are a specific class of C_{35} or C_{37} secondary metabolites derived from the polyketide pathway.[7] Since the isolation of the first acetogenins in 1982, the structures and pharmacological activities of this interesting class of natural products have been subjected to intensive investigations. Different acetogenins have proved to exhibit several biological functions such as cytotoxicity and in vivo antitumor, pesticidal, antimalarial, anthelmintic, piscicidal, antiviral, and antimicrobial activities. Despite the wide spectrum of biological activities studied, the anti-inflammatory effect of the acetogenins has not been reported.[8−10]

In this study, 15 typical acetogenins (**1**−**15**) from our laboratory were investigated for their inhibition potency of COX-2 expression by real-time PCR. The compounds tested can be classified into three main categories based on the number and position of tetrahydrofuran (THF) rings in the structure. Acetogenins with adjacent bis-THF rings included isodesacetyluvaricin (**1**),[11−14] desacetyluvaricin (**2**),[13−15] squamocin (**3**),[14,16] asimicin (**4**),[14] bullatacin (**5**),[14] squamocin O_2 (**6**),[17] and squamocin O_1 (**7**).[17] Bullatalicin (**8**)[18] belongs to the nonadjacent bis-THF type. On the other hand, compounds with a mono-THF ring were annonacin (**9**),[19−21] corossoline (**10**),[21,22] annomontacin (**11**),[23] uvariamicin III (**12**),[24] muricin H (**13**),[25] glaucafilin (**14**),[26] and arianacin (**15**).[27] Screening results showed that compound **1**, with two flanking hydroxy groups, both in a *threo* configuration, exhibited the most potent inhibitory activity in the bioassay used. Isodesacetyluvaricin (**1**) was isolated from a MeOH extract of *Annona glabra* L.

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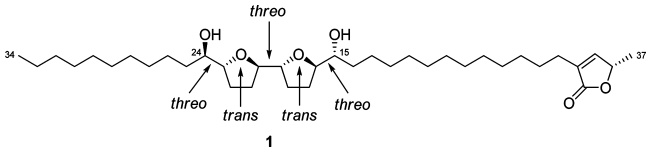
(Annonaceae). The effect of **1** was tested on COX-2 mRNA and protein levels, and its molecular mechanisms of COX-2 gene expression, cell proliferation, and intracellular calcium signaling were also probed. Evidence is reported herein that the inhibitory properties of **1** make it a promising candidate to counteract the pathological effects of COX-2 overexpression in cells.



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RESULTS AND DISCUSSION

Isodesacetyluvaricin (1) Does Not Affect EGF-Stimulated Cell Proliferation. To test for the possible toxicity of **1**, the effects of using 1 μM or 5 μM **1** were evaluated on epidermal growth factor (EGF)-stimulated proliferation of the A431 epidermoid carcinoma cell line. Treatment of cultures with EGF at 25 ng/mL stimulated cell proliferation, but the addition of **1** had no significant effect after 24 and 48 h (Figure 1A, B).

Isodesacetyluvaricin (1) Inhibits Expression of COX-2 but Not COX-1. To analyze the anti-inflammatory effects of **1**, A431 carcinoma cells were used, since they have an abundance of EGF receptors on the plasma membrane. Treatment with EGF at 25 ng/mL increased the amount of COX-2 mRNA (Figure 2A). Addition of **1** resulted in a dose-dependent inhibition of COX-2 transcription without a corresponding change in the amount of COX-1 mRNA. Similar results were obtained with the Raw 264.7 mouse leukemic monocyte/macrophage cell line. In these leukemic cells, compound **1** inhibited COX-2 expression stimulated by lipopolysaccharide (LPS) at 10 μg/mL (Figure 2B). To confirm these findings, a plasmid that encoded the COX-2 gene promoter was used. Compound **1** reduced significantly EGF-induced COX-2 gene promoter activity in A431 cells (Figure 2C). In addition, EGF-induced COX-2 protein expression in A431 cells was reduced by **1** in a dose-dependent manner (Figure 2D). Importantly, compound **1** showed stronger inhibitory effects against the COX-2 gene than indomethacin, a therapeutically available COX inhibitor (Figure S1, Supporting Information).

Mechanism of COX-2 Inhibition by Isodesacetyluvaricin (1). Evidence indicates that binding sites for the cAMP response element binding factor (CREB) and the nuclear factor of activated T cells (NFAT) participate in EGF-mediated transcriptional activation of COX-2.[28−31] In A431 cells, the results obtained confirmed the functional roles of CRE and NFAT by designed plasmids with deletion and mutation (Figure S2, Supporting Information). Luciferase assays were used to test the inhibitory effect of **1** on COX-2 promoter binding sites of CREB and NFAT. At concentrations ranging from 1 to 5 μM, the acetogenin **1** reduced the relative promoter activity of CREB (Figure 3A) and NFAT (Figure 3B). The results are consistent with the concept that compound **1** can influence directly the activities of CREB and NFAT. However, it remains possible that inhibition is indirect and may involve kinases and phosphatases that regulate the capacity of CREB and NFAT binding to the COX-2 promoter. Relevant reactions may include phosphorylation of CREB by protein kinase A-dependent Rap1-extracellular-signal-related kinase and dephosphorylation of NFAT by calcineurin.[32−34]

Isodesacetyluvaricin (1) Does Not Affect Intracellular Calcium Signaling. CREB and NFAT are calcium-dependent transcription factors.[35] To determine whether **1** inhibits EGF-stimulated expression of COX-2 by impeding entry of store-operated calcium, intracellular calcium signals were monitored in a single A431 cell. It was found that thapsigargin evoked store-operated calcium influx (Figure 4A, blue line). However, pretreatment with compound **1** at a concentration of 1 μM did not interfere with thapsigargin-stimulated calcium signals (Figure 4A, red line). Calculations of the relative amount (Figure 4B) of store-operated calcium influx showed that there were no significant differences in the signals from calcium entry. These results do not support the inhibitory effects of **1** as being due to a diminished concentration of intracellular calcium. Thus, compound **1** inhibits EGF-stimulated COX-2

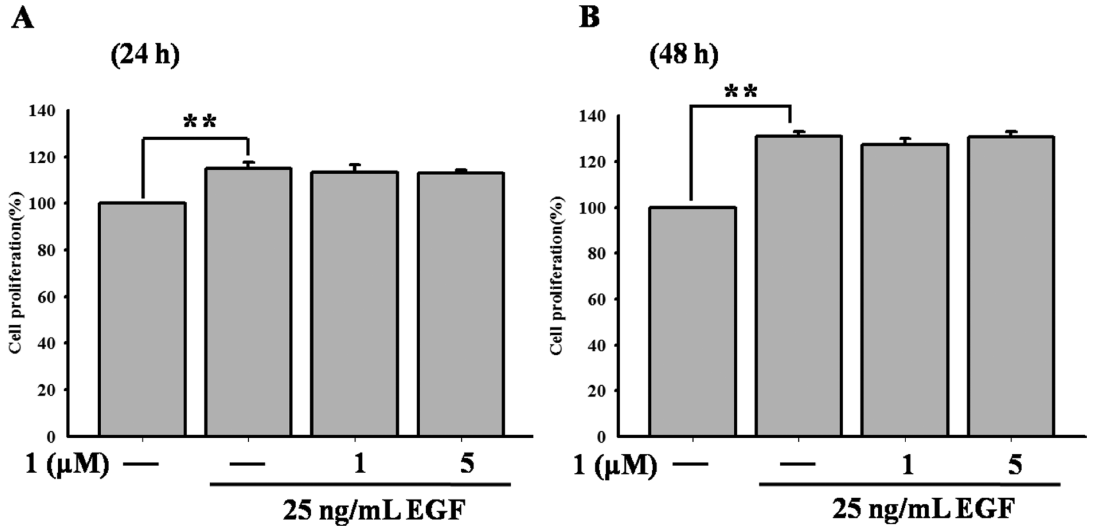

Figure 1. Isodesacetyluvaricin (**1**) does not affect EGF-stimulated cell growth. A431 cells were seeded into each well of a 24-well plate and incubated at 37 °C for 24 h. Cells were pretreated with **1** at 1 μM or 5 μM for 30 min and then stimulated by EGF at 25 ng/mL for 24 h (A) or 48 h (B). Cell growth was measured using the WST-1 assay. Statistically significant data are indicated by * for $p < 0.05$ and ** for $p < 0.01$.

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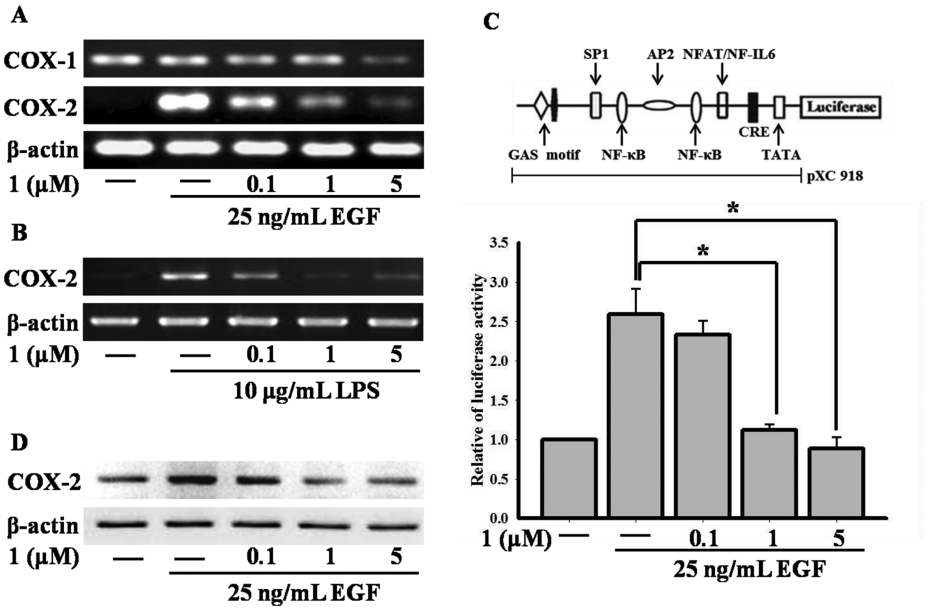

Figure 2. Isodesacetyluvaricin (**1**) inhibits COX-2 expression in a dose-dependent manner. (A) A431 cells were pretreated with 0.1, 1, or 5 μM **1** for 30 min. Then, cells were stimulated with EGF at 25 ng/mL for 3 h. Total RNA was extracted from the cells, and expression of COX-1 and COX-2 mRNAs was measured by RT-PCR. (B) Raw 264.7 cells were pretreated with 0.1, 1, or 5 μM **1** for 30 min. Then, cells were exposed to LPS at 10 μg/mL for 3 h. Total RNA was extracted from the cells, and expression of COX-2 mRNA was measured by RT-PCR. (C) A431 cells were transiently transfected with 0.5 μg of COX-2 promoter plasmid pXC 918 for 24 h. After treatment with **1** followed by stimulation with EGF, luciferase activity and total cell lysates concentrations were determined and normalized. Values for luciferase activity are means ± SEM. Statistical significance (*p < 0.05 and **p < 0.01) of the differences between the results was analyzed by Student's t-test. (D) After A431 cells were pretreated with **1** followed by stimulation with EGF, total cell lysates were prepared. Western blotting was performed to detect COX-2 protein.

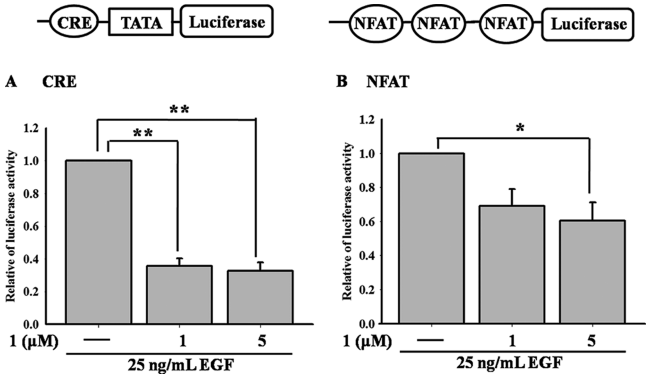

Figure 3. Isodesacetyluvaricin (**1**) inhibits EGF-mediated activation of the COX-2 promoter by CREB and NFAT. A431 cells were transfected transiently with (A) 0.5 μg of plasmid pXC 80 that encoded the CRE promoter site or (B) the NFAT promoter site. After 24 h, cells were pretreated with **1** at 1 or 5 μM for 30 min and were then stimulated with EGF at 25 ng/mL for 3 h. Luciferase activities and total cell lysate concentrations were determined and normalized. Values for luciferase activity are means ± SEM. Statistical significance (*p < 0.05 and **p < 0.01) of the differences between the results were analyzed by Student's t-test.

expression by suppressing the effects of NFAT and CREB on the gene promoter, and it is unlikely to function as a store-operated calcium channel blocker (Figure S3, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus (Thermo Fisher Scientific Inc., Rockford, IL), and the values presented are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter (JASCO, Inc., Tokyo, Japan). UV spectra were obtained on a JASCO V-530 UV/vis spectrophotometer (JASCO, Inc.). The IR spectra were measured on a Mattson Genesis II spectrometer (Mattson Instruments, Madison, WI, USA). ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity Plus-400 and Varian Gemini-200 MHz NMR spectrometers (Varian Inc., Palo Alto, CA, USA). Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in hertz. LRESIMS were measured on a VG Biotech Quattro 5022 mass spectrometer (VG Biotech Ltd., Altrincham, UK). Silica gel 60 (Merck, 230−400 mesh, Merck KGaA, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich Corp., St. Louis, MO, USA) were used for column chromatography, while TLC analysis was carried out on silica gel GF_{254} precoated plates (Merck KGaA), with detection performed using 50% H_2SO_4, followed by heating on a hot plate. HPLC isolation was performed with a Hitachi L-7100 series HPLC (Hitachi Inc., Tokyo, Japan), equipped with a Bischoff RI detector and a Shimadzu LC-10AT series HPLC (Shimadzu Inc., Kyoto, Japan), with a SPD-10A UV/vis detector or photodiode array detector. ODS (Hypersil, 250 × 10 mm i.d., 5 μm, Thermo Fisher Scientific Inc.) columns were utilized for HPLC separation.

Chemicals. The structures of the compounds tested in this investigation were elucidated and identified based on spectroscopic analysis and compared with literature values as follows: isodesacetyluvaricin (**1**), desacetyluvaricin (**2**), squamocin (**3**), asimicin (**4**), bullatacin (**5**), squamocin O_2 (**6**), squamocin O_1 (**7**), bullatalicin (**8**), annonacin (**9**), corossoline (**10**), annomontacin (**11**), uvariamicin III (**12**), muricin H (**13**), glaucafilin (**14**), and arianacin (**15**). The purity of all acetogenins was >97%, which was assessed with a three-point peak purity method and determined using Shimadzu "Class VP" software. Among these compounds, isodesacetyluvaricin (**1**) showed the most potent anti-inflammatory activity and was selected for further investigation. Presented below are extraction and isolation procedures for **1**.

Plant Material. The seeds of *A. glabra* were collected from Tai-Tung City, Taiwan, in March 2007. The species was identified by Dr. Ming-Hong Yen, Graduate Institute of Natural Products, Kaohsiung

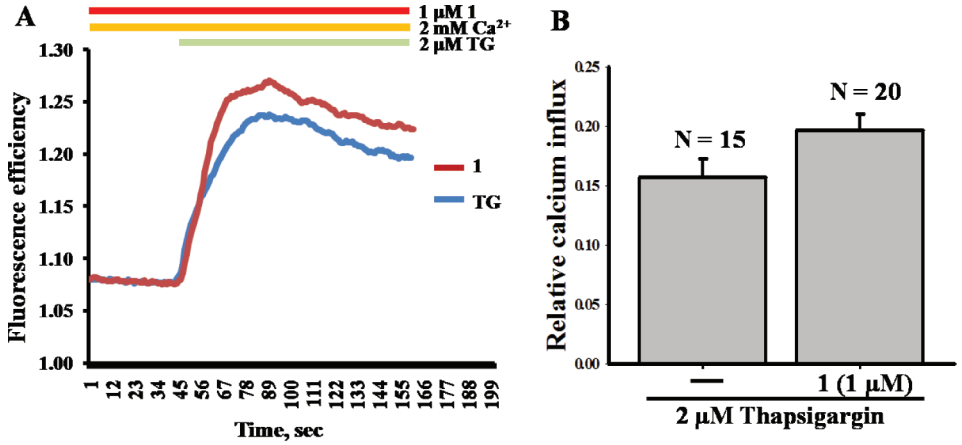

Figure 4. Isodesacetyluvaricin (**1**) does not affect store-operated Ca^{2+} entry. (A) Fluorescence-based calcium assay showed that thapsigargin (TG) at 2 μM evoked an influx of Ca^{2+} (blue line). Pretreatment with **1** at 1 μM did not diminish the influx (red line) in A431 cells. (B) Aggregate data were pooled from 15 cells exposed to thapsigargin alone or from 20 cells pretreated with **1**. Treatment with **1** did not reduce the relative amount of calcium influx.

Medical University, Kaohsiung, Taiwan. A voucher specimen (Annona-02) was deposited at the Graduate Institute of Natural Products, Kaohsiung, Taiwan.

Extraction and Isolation of Isodesacetyluvaricin (1). Seeds of *A. glabra* (1.1 kg) were extracted with methanol (5 L × 5). The MeOH extract (106.6 g) was partitioned between *n*-hexane and 80% MeOH(aq) to yield *n*-hexane and 80% MeOH(aq) extracts. After removing solvent in vacuo, the 80% MeOH(aq) residue (75.0 g) was partitioned between *n*-butanol and H_2O to yield *n*-butanol (38.3 g) and H_2O (23.0 g) layers. The *n*-butanol layer was further separated into six fractions by column chromatography on Diaion HP-20 gel with gradient elution using H_2O–MeOH (1:0, 7:3, 4:6, 2:8, 0:1) and acetone. Fraction 5 (15.1 g) was subjected to column chromatography on silica gel (200–300 mesh), eluted with *n*-hexane–$CHCl_3$–MeOH (gradient), to obtain 14 fractions. Fraction 5-6 (382.1 mg) was further isolated and purified by preparative reversed-phase HPLC [Thermo Hypersil ODS column, 250 × 10 mm, with gradient elution of MeOH–H_2O (86:14–100:0), flow rate 4.7 mL/min; UV detector set at 220 nm] to obtain **1** (54.7 mg, t_R 28.5 min). This compound exhibited physical ($[\alpha]_D^{25}$) and spectroscopic (UV, IR, NMR, MS) data consistent with literature values for isodesacetyluraricin (**1**).[11−14]

Cell Culture. EGFR-rich A431 cells and the Raw 264.7 cells were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen Corp.) and 100 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen Corp.). For each experiment, cells were grown in serum-free DMEM medium for 24 h before treatment with **1**.

Cellular Proliferation Assay (WST-1). A431 cells were pretreated for 30 min with **1** and then exposed to EGF (25 ng/mL) for 24 or 48 h. To assay cell proliferation, cells were incubated with WST-1 reagent (Roche, Indianapolis, IN, USA) at 37 °C for 5 to 10 min. An ELISA reader was used to measure the absorbance of the samples at 450 nm with 600 nm as the reference wavelength.

DNA Transfection and Luciferase Assay. A431 cells were plated in six-well plates for 24 h. Then, the cells were transiently transfected with 0.5 μg of COX-2 promoter plasmid in 1 mL of Opti-MEM medium (Invitrogen Corp.) containing 1 μL of Lipofectamine 2000 (Invitrogen Corp.). After pretreatment with or without **1**, luciferase activity of the COX-2 reporter was measured using a dual-luciferase reporter assay (Promega, Madison, WI, USA). Data were normalized to the protein concentration of each well.

Reverse Transcription PCR. Total RNA was isolated from A431 cells and Raw 264.7 cells with Trizol reagent (Invitrogen Corp.), according to the manufacturer's instructions. Reverse transcriptase reactions were performed on 1 μg samples of RNA using a reverse transcription kit (Invitrogen Corp.). Incubation conditions included 10 min at 25 °C, 120 min at 37 °C, or 5 min at 85 °C. The resulting cDNAs were used to detect COX-1 and COX-2 expression levels by reverse transcription PCR (RT-PCR).

PCR and DNA Gel Electrophoresis. After synthesis of cDNA, the following gene-specific primers were used: COX-1 (207 bp), forward primer: CCT CAT GTT TGC CTT CTT TGC; reverse primer: GGC GGG TAC ATT TCT CCA TC; human COX-2 (221 bp), forward primer: ATC AAT GCA AGT TCT TCC CGC; reverse primer: GAC TCC TTT CTC CGC AAC A; human β-actin (145 bp), forward primer: ATC TCC TTC TGC ATC CTG TCG GCA AT; reverse primer: CAT GGA GTC CTG GCA TCC ACG AAA C; mouse COX-2 (860 bp), forward primer: GGA GAG ACT ATC AAG ATA GTG ATC; reverse primer: ATG TCA GTA GAC TTT TAC AGC TC; mouse β-actin (603 bp), forward primer: GTG GGC CGC CCT AGG CAC CAG; reverse primer: GGA GGA AGA GGA TGC GGC AGT. After denaturing the DNA at 94 °C for 5 min, 35 cycles of amplification were performed: 94 °C 30 s, 58 °C 30 s, 72 °C 1 min. Then, the reaction was incubated at 72 °C for 7 min. PCR products were separated by gel electrophoresis in 2% agarose gel using TAE buffer and visualized by ethidium bromide staining and UV transillumination.

Extraction of Proteins and Western Blotting. Total cell lysates (40 μg) were isolated in RIPA buffer containing proteinase inhibitors and analyzed by SDS-PAGE on a 12.5% gel. After electroblotting to nitrocellulose, membranes were blocked with 5% nonfat dry milk for 1 h at room temperature. Then, membranes were washed three times with 0.1% PBST for 10 min at room temperature. Membranes were incubated with working dilutions of mouse monoclonal primary antibodies for 16 h at 4 °C. The dilution of antibody to COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was 1:2000. The dilution of antibody to β-actin (Santa Cruz Biotechnology) was 1:20 000. Membranes were washed three times with 0.1% PBST and incubated with a 1:5000 or 1:10 000 dilution of anti-mouse IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Finally, membranes were washed three times with 0.1% PBST, and the protein bands were visualized using an ECL-Plus Western blotting detection system (Millipore Corp., Bedford, MA, USA).

Determination of Calcium Concentration. A431 cells were seeded onto glass coverslips for 24 or 48 h. Then, the attached cells were loaded with 1 μM Fluo-4 (Invitrogen Corp.) at 37 °C for 20 min in the dark. Cells were washed three times in standard external solution: 145 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4. Changes in fluorescence intensity of Fluo-4 in loaded cells were detected by time-lapse video-microscopy (Olympus IX70, Olympus Optical Co., Ltd., Tokyo, Japan) and analyzed by the cell $^{\Lambda}$R system (Olympus Optical Co., Ltd.).

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Statistical Analysis. Student's *t*-test was used to compare all experimental results. *p*-Values less than 0.05 were considered significant and are denoted by *. Highly significant *p*-values less than 0.01 are denoted by **.

■ ASSOCIATED CONTENT

Supporting Information

Structures of tested acetogenins; Western blot analysis of LPS-stimulated COX-2 mRNA expression in Raw 264.7 cells for **1** and indomethacin (Figure S1); luciferase assay results of **1** (Figure S2); schematic representation of COX-2 inhibition signaling pathways (Figure S3). This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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